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Assessing the Vulnerability of Zooplankton Populations Following an Environmental Disturbance History

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Abstract

Environmental disturbances can have lasting impacts resulting on aquatic vulnerable populations, therefore influencing their capacity to cope with subsequent stressors. This protocol offers a method to assess the vulnerability of aquatic populations, using *Daphnia magna* as a model organism, with a previous history of environmental disturbance characterized by the exposure to sublethal concentrations that are generally assumed to not have impact on aquatic communities (i.e., exposure to agrochemicals concentrations within legal limits). Using controlled laboratory exposures over several generations, populations previously subjected to sublethal chemical exposures were challenged with subsequent environmental stressors (i.e. resource limitation). Findings from this vulnerability assessment protocol indicate that prior environmental disturbances can compromise population responses, increasing susceptibility to stress. This method has the advantage of being easily applicable to other species and to both chemical and, after adjustment and testing, to non-chemical disturbances of interest potentially driving vulnerability. The methods' adaptability increases their usefulness and enables broad applications in assessing the impact of an environmental disturbance history on zooplankton populations. A disadvantage is the difficulty in determining sublethal concentrations as it depends on multiple biological and abiotic factors. This challenge can be overcome by defining a clear selection criterion based on reference thresholds accepted both scientifically and by regulators to set environmental quality standards and permissible exposure limits.

Environmental disturbance history. In the context of aquatic ecotoxicology under the framework of population vulnerability assessment, it focuses on how previous exposure to sublethal chemical exposures shape population responses to subsequent stressors.

Sublethal concentrations. Sublethal concentrations denote exposure levels to a toxicant that do not result in mortality, but can lead to long-term detrimental effects on populations physiology and behavior. In the vulnerability induction phase, the No Observed Effect Concentration (NOEC) is used to define the sublethal concentration for the environmental disturbance history treatment. While the NOEC is often used in toxicity studies to define safe exposure levels that prevent population mortality, it may fail to account for vulnerability shifts that can lead to later mortality.

Vulnerability induction. It is the phase where a population is reared under a continuous sublethal chemical exposure which might result on an increase of population susceptibility to subsequent stressors (e.g., resource scarcity or other chemical exposures).

Vulnerability verification: starvation test. The starvation test is used as a stress assay to assess differences in survival time under food deprivation between populations with a disturbance history (i.e. previous sublethal chemical exposure) and control populations without disturbance history (i.e. optimal culture conditions).

Figure 1. Glossary of key terms employed in the protocol.

Guidelines

General considerations

This protocol assumes prior experience with culturing *D. magna* and experience in standard protocols for ecological risk assessment.

Safety notes

All chemicals must be handled and disposed of properly, even at sublethal concentrations, in accordance with national regulations and institutional standards.

Future directions

The protocol has the potential to be expanded to other species and stressors.

Materials

Chemical of study
Material to prepare stock solutions
Culture chamber
D. magna cultures
Green algae
Beakers or aquariums for the *D. magna* cultures, exposure phase and later stress test
Counters
Material according to the selected stress tests

Vulnerability induction: environmental disturbance history under sublethal chemical exposure

1 **Goal**

The first phase of the vulnerability assessment protocol is designed to establish a controlled history of environmental disturbance in *D. magna* populations through sublethal exposure to a chemical stressor of ecological relevance in aquatic pollution (Fig. 1 and Fig. 2). The relevance of this phase lies in its ecological realism. Many aquatic populations are not exposed to short intensive pollution events but rather to chronic and sublethal chemical concentrations. The evaluation of these scenarios falls outside the focus of regulatory ecological risk assessments. Experiments considering the history of sublethal environmental disturbance allow to evaluate how hidden effects of past disturbances alter population responses to subsequent stressors increasing the realisms of ecological risk assessments.

2 **Sublethal exposure criteria**

Sublethal exposures are frequently encountered in surface waters, occurring at concentrations below regulatory thresholds yet still ecologically relevant. These concentrations do not result on aquatic invertebrates mortality, however chronic exposure can have subtle but important effects over generations. Consequently, inducing vulnerable populations with lower capacity to respond to subsequent stressors (Fig. 1). Sublethal exposure criteria combines both working below lethal (i.e., $LC_{50,}$ López-Valcárcel et al., 2021) or below observed concentration with an impact on biological endpoints (i.e., NOEC, López-Valcárcel et al., 2025, 2024, 2023) together with selecting chemical concentrations that are environmental relevant aiming to provide a realistic evaluation of ecological risk in water bodies where populations are exposed to chemicals without showing immediate signs of collapse.

3 **Cladocera population and generations under sublethal chemical exposure**

First of all, individuals to start the experiment with eggs need to be isolated from the laboratory culture of *D. magna* with the aim of obtaining enough newborns to initiate the history of environmental disturbance phase. The individuals are kept for 5 days at 20°C and a cycle of 12:12 h light:dark (it can be adapted to other laboratory conditions, *D. magna* clones or other species) in a controlled chamber in glass aquariums (2 L) and reared in mineral water enriched with thiamine (0.075 g/L), vitamin B12 (0.010 g/L), biotin (0.0075 g/L) and sodium selenite (0.010 g/L). As a food resource, we used the microalgae *Scenedesmus obliquus* (Turpin) Kützing 1833 ad libitum.

Second, populations are assigned to either control treatments without chemical exposure or to treatments with sublethal chemical concentrations (as defined by the sublethal exposure criteria above) and reared under these conditions for multiple generations, at least two. The experiment is carried out under controlled laboratory conditions, with temperature maintained at 20 °C and a light-dark cycle of 12:12 h to replicate optimal culture conditions. Populations are fed ad libitum daily with green algae to avoid confounding effects of food limitation. The medium is renewed every 7 days to maintain water quality and constant chemical concentration. Chemical concentrations should be measured to verify that the nominal values correspond to the intended target concentrations

During the sublethal chemical exposure phase, which extends across several *D. magna* generations (e.g., 21 days, López-Valcárcel et al., 2021), several biological endpoints can be recorded to characterize each population status (i.e., life-history traits such as time to first reproduction, clutch size and total offspring per female). Specifically, the main goal of this phase is to obtain population lineages with diverse history of environmental disturbance varying in environmental realistic sublethal exposures together with control populations representing unpolluted environments. This goal is based on the hypothesis that, although populations may survive and reproduce under continuous sublethal chemical exposures at concentrations assumed to be ecologically safe, such exposure compromises their ability to withstand subsequent stress.

Vulnerability verification: starvation test

4 After the environmental disturbance history phase using sublethal chemical exposure the aim is to assess the vulnerability of *D. magna* population to subsequent stressors (Fig. 1). Consequently, the results will test the hypothesis that, although populations may survive and reproduce under continuous sublethal chemical exposure at concentrations considered environmentally safe, such exposure compromises their capacity to withstand subsequent stress. The main stress test used has been a test for inanition because food scarcity represents a common and ecologically relevant stressor in aquatic ecosystems that frequently overlaps with chemical exposures. In this protocol, we will present the starvation test (López-Valcárcel et al., 2021). Other stress tests can be performed depending on the research question of interest, for instance, in a context of: a) global change, factors as temperature or salinity can be of interest (López-Valcárcel et al., 2023); b) pharmaceutical products pollution, subsequent exposure test to medicaments are relevant (i.e. paracetamol, López-Valcárcel et al., 2024); c) chemically fragmented habitats, scape behavioural test are applicable to evaluate changes in species dispersal patterns (López-Valcárcel et al., 2025).

Experimental design overview

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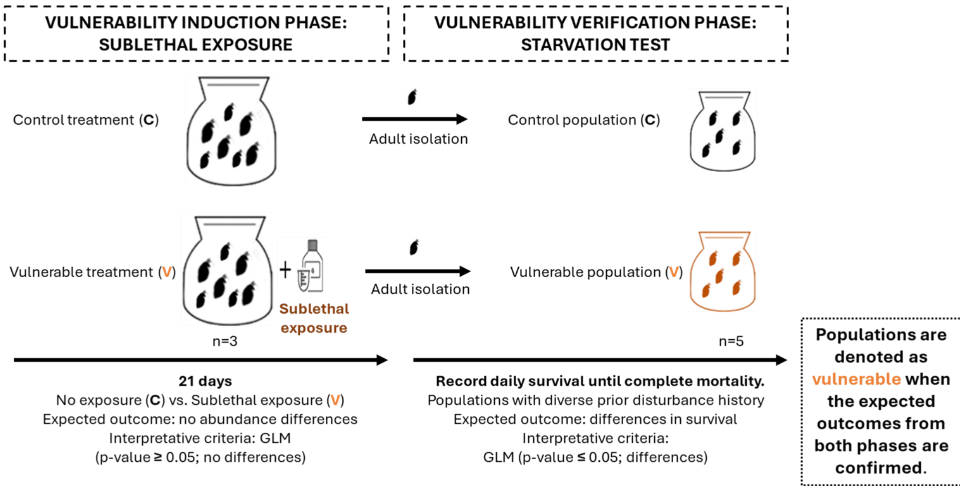

Figure 2. Scheme of the experimental phases to evaluate *D. magna* population vulnerability.

VULNERABILITY INDUCTION

The aim of this phase is to generate a vulnerable population of *D. magna* (or another suitable model organism) through prolonged exposure to a sublethal concentration of a commonly used chemical (i.e., agrochemicals such as herbicides, insecticides, or fungicides). Specifically, these sublethal exposures may increase population susceptibility to subsequent stressors, which will later be evaluated during the vulnerability verification phase, yet the **expected outcome** is to find **no differences in *D. magna* abundance** between non-exposed (control) and exposed (vulnerable) populations which mask impact at different biological levels (i.e. physiological responses).

Experimental setup

1. Microcosm preparation

Use glass aquaria or containers of 2 L capacity, maintained at 20 ± 1 °C under a 12:12 h light–dark photoperiod.

2. Feeding conditions

Provide *S. obliquus* ad libitum as the only food source throughout the experiment. The algal culture should be grown in a standardized nutrient medium (e.g., 3N-BBM*V).

3. Treatment establishment

Set up two **treatments**, each with three replicates:
Control (C): population maintained in clean medium without chemical.
Vulnerable (V): population continuously exposed to a sublethal concentration of the selected chemical, determined in reference to its NOEC values reported in the literature or obtained from preliminary range-finding tests (see "Sublethal exposure criteria" section above).
4. Exposure period
Maintain both treatments for 21 days, rearing several generations of *D. magna* under control conditions (i.e. no exposure) *vs.* an environmental disturbance history (i.e. sublethal exposure).
5. Medium renewal
Renew the medium once per week, restoring the initial concentration of the chemical to ensure stable exposure conditions.
6. Monitoring parameters
Record weekly: Abundance of individuals (ind/L). Physicochemical variables: pH and dissolved oxygen (DO).
7. **Interpretative criteria:** A lack of *D. magna* abundance response may mask effects occurring at other biological levels. Verify that there are no significant differences in abundance between C and V populations. And, that physicochemical parameters are not altered confirming suitable culture conditions. The absence of lethal effects confirms that the concentration used is sublethal. However, it might be sufficient to create a previous environmental disturbance history leading to vulnerability.

VULNERABILITY VERIFICATION: STARVATION TEST
The aim of this phase is to verify whether previous sublethal exposure to the chemical reduces the physiological tolerance of the organisms to a new stressor, specifically food deprivation. The **expected outcome** is to find **differences in *Daphnia magna* survival** under subsequent stressors following the prior sublethal exposure phase.

Experimental setup
1. Adult collection
Isolate adult individuals from both **C** and **V populations**. Assign them according to their parental treatment.
2. Experimental units
Place five adults in 50 mL containers, using five replicates per treatment.
3. Experimental conditions
Maintain all units at 20 ± 1 °C under a 12:12 h light–dark photoperiod. Do not provide food during the entire experimental period.
4. Duration and observations
Monitor the individuals daily until 100% mortality occurs in both treatments.
5. Data collection
Record daily survival of each individual. Calculate the number of survival days per organism and the mean survival time for each treatment.

6. Data analysis
Compare survival between treatments using statistical tests (e.g., generalized linear models, GLM).
7. **Interpretative criteria:** a significant reduction in survival days in the Vulnerable population relative to the Control confirms that prior sublethal exposure to the chemical has induced physiological vulnerability, reducing the ability of the organisms to cope with additional stress. Therefore, it verifies the vulnerability of the population.

Statistical analysis

6 Statistical analyses for assessing the vulnerability of aquatic populations with a history of environmental disturbance aims to compare control and exposed populations in order to determine whether prior exposure increases population vulnerability. We have chosen GLM because it allows for the analysis of different outcome distributions and variance patterns, providing a better fit for the characteristics of our data (Zuur et al., 2009). Comparisons using GLM between control and exposed population are done at the end of the two experimental phases (Vulnerability Induction phase and Vulnerability Verification phase) and evaluated using the interpretative criteria.

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